REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
		FINAL 01 Dec 94 TO 30 Nov 95

4. TITLE AND SUBTITLE
IN VITRO APPROACH TO PREDICTIVE TOXICOKINETICS

5. FUNDING NUMBERS
F49620-95-1-0104
61102F
2312/AS

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AFOSR-TR-96
0123

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Dr Walter Kozumbo

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release;
distribution unlimited.

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

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19960320 042

14. SUBJECT TERMS			15. NUMBER OF PAGES
			16. PRICE CODE
17. SECURITY CLASSIFICATION OF REPORT (U)	18. SECURITY CLASSIFICATION OF THIS PAGE (U)	19. SECURITY CLASSIFICATION OF ABSTRACT (U)	20. LIMITATION OF ABSTRACT (U)

Grant Number: F49620-95-1-0104

FINAL REPORT - IN VITRO APPROACH TO PREDICTIVE TOXICOKINETICS

31 JAN 96

P.I. - JOHN M. FRAZIER, PH.D.

WRIGHT STATE UNIVERSITY
Research and Sponsored Projects
3640 Colonel Glenn Highway
Dayton, OH 45435

OBJECTIVES:

The overall objective of this research program is to develop new approaches to predict the systemic toxicokinetics of chemicals utilizing *in vitro* experimental model systems and biologically-based kinetic (BBK) models. The focus of the proposed research is to test the hypothesis that *in vitro* measurements of membrane transport parameters in hepatic membrane vesicles are predictive of hepatic transport *in vivo*. Although this study is focused on transport processes in one particular organ, i.e. hepatic transport processes, these processes are a major controlling factor in determining systemic kinetics of many chemicals, particularly those which do not satisfy the "perfusion limited" condition usually assumed in many current PBPK models.

The first phase of the proposed three year research project is to develop a predictive paradigm for hepatic transport kinetics in the intact animal based on the rat hepatic membrane vesicle model. The specific goals are:

(1) To investigate the transport kinetics of a "set of reference chemicals" (cadmium, benzoic acid and trichloroacetic acid) in the rat hepatic sinusoidal membrane vesicle model and derive transport parameters for these chemicals,

(2) To utilize the isolated perfused rat liver model to determine scaling factors to convert transport parameters derived from the vesicle model (P = mass transport per unit concentration per unit area of membrane per unit time) to appropriate parameters for tissue kinetics in the BBK model (PA = mass transported per unit concentration per unit time),

(3) To incorporate the experimentally derived kinetic parameters into a BBK model for the rat and simulate *in vivo*, systemic kinetics, and

(4) To compare predicted kinetics with experimental, *in vivo*, rat kinetic data to refine the predictive paradigm.

Having established the predictive paradigm, the primary hypothesis will be tested by determining the membrane transport properties of a "second set of test chemicals" (phthalic acid, catechol and acetylsalicylic acid) in the hepatic membrane vesicle model and using the paradigm to predict *in vivo* kinetics. Both qualitative and quantitative criteria will be used to test goodness of fit.

This project is the first step in a series of planned studies to develop techniques to incorporate transport parameters derived from various *in vitro* models into BBK models. The long range goal is to utilize these techniques to predict *in vivo* toxicokinetics of chemicals in humans. The rat studies described here using *in vitro* models to predict *in vivo* toxicokinetics form the basis for the *in vitro* approach to predicting human kinetics.

STATUS OF EFFORT

Dr. Frazier, the Project Director for F49620-95-1-0104, was appointed to an Air Force ST position in November, 1995. He is stationed at OL AL HSC/OET, WPAFB. Consequently, his AFOSR project, administered through Wright State University, was officially transferred to AL/OET and combined with the Predictive Toxicology project (2312A202) already in place at AL/OET (previously administered by Dr. Jeff Fisher). Dr. Frazier is now the Project Director for the combined project. This report constitutes the final report for the original project. Subsequent progress reports will encompass the combined Predictive Toxicology project. The scope of both projects remain unchanged.

This progress report will discuss developments since the last annual progress report provided in December 1995 (see Appendix 1).

ACCOMPLISHMENTS/NEW FINDINGS

The main accomplish during this period is the completion of trichloroacetic acid (TCA) kinetic studies in the IPRL system. Rat livers were isolated from 200 g Fischer 344 rats and incubated in the computer controlled perfusion apparatus for a control period of 1 h. At the end of the control incubation, radiolabelled TCA was added to the perfusion medium (PM) to an initial concentration of 250 μM. This concentration was selected to correspond to the initial plasma concentration observed in vivo when rats were injected i.v. with 10 mg/kg TCA. The kinetic of TCA were observed for a period of 2 h by collecting perfusion medium samples at 2, 5, 10, 15, 30, 60, 90, 120 min and detecting TCA associated radioactivity by liquid scintilation counting. Bile was also collected over 30 min intervals throughout the incubation and assayed for TCA associated radioactivity. At the end of the 2 h incubation in the presence of TCA, the liver was removed, homogenized and assayed for TCA. Liver tissue samples were taken for histopathology (both light and electron microscopy). No evidence of TCA toxicity at the morphological level was observed.

The experimental data suggest that TCA rapidly reaches a dynamic equilibrium between the PM and the liver tissue. Throughout the remainder of the study the concentration in the incubation medium remained constant. This observation was not anticipated since TCA is a charged molecule at pH 7.4 and was expected to exhibit diffusion limited characteristics. The observed distribution between the PM and the liver suggested that protein binding may be playing a role in the dynamic equilibrium established (see below). TCA was eliminated in the bile. The rate of elimination in the bile decreased over the 2 h incubation with the highest amount eliminated during the first 0.5 h. The total amount eliminated in the bile over the 2 h incubation was less than 1 % of the administered dose. The concentration of TCA in the liver was determined at the end of the experiment and was lower than predicted assuming a totally passive distribution between PM and intracellular water spaces. Again this suggests protein binding in the PM may be important.

The results obtained to date suggested several additional studies. First, the issue of protein binding is being investigated. Preliminary data from protein binding studies using centrifigal

ultrafiltration techniques indicate a significant fraction of the TCA in PM is bound to protein. The only protein present in the PM is bovine serum albumin (BSA), which is presumably responsible for the effect. Using a Scatchard analysis, the binding capacity and binding affinity for the TCA-BSA complex has been estimated. Further studies investigating TCA binding in rat plasma and in liver tissues are scheduled. Additional studies designed to explain the decrease in biliary elimination of TCA over the course of the experiment are underway. Elimination of bromosulfophthalein (BSP), a well studied chemical that is excreted in the bile, will be investigated in the IPRL to determine whether the decrease in biliary elimination is an artifact of the experimental preparation. If this possibility is ruled out, we will investigate whether TCA itself modulates biliary excretion of BSP. This would determine whether TCA specifically inhibits transport of chemicals into bile. We already know that TCA has no effect on formation of bile. It is anticipated that these studies will take several weeks, but the results will allow us to better understand the IPRL preparation and improve our ability to interpret kinetic data derived from this *in vitro* model system

INTERACTIONS/TRANSITIONS

Poster Presentation SOT Annual Meeting, March, 1996 - Frazier, J.M. and Toxopeus, C. A biologically based kinetic model for the isolated perfused rat liver. (Appendix 2)

APPENDIX 1

Grant Number: F49620-95-1-0104

IN VITRO APPROACH TO PREDICTIVE TOXICOKINETICS

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This project is the first step in a series of planned studies to develop techniques to incorporate transport parameters derived from various *in vitro* models into BBK models. The long range goal is to utilize these techniques to predict *in vivo* toxicokinetics of chemicals in humans. The rat studies described here using *in vitro* models to predict *in vivo* toxicokinetics form the basis for the *in vitro* approach to predicting human kinetics.

STATUS OF EFFORT

This project was operationally initiated in December, 1994. Due to the move of the P.I. from Johns Hopkins University to Wright State University/WPAFB, recruitment of new laboratory personnel to conduct experimental studies has been a rate limiting factor. Currently, one postdoctoral fellow (Corike Toxopeus, Research Institute of Toxicology, University of Utrecht) has been recruited and has been working in the laboratory since June 21, 1995. Additional personnel are actively being recruited.

The focus of the first six months of research efforts has been dictated by the availability of personnel. The decision was made to address aspects of the project that could be successfully accomplished with limited resources. Specific tasks undertaken were:

(1) Developing a biologically-based kinetic (BBK) model for water soluble diffusion limited chemicals,

(2) Conducting *in vivo* kinetic studies on one of the test chemicals (trichloroacetic acid).

(3) Developing a BBK model for the isolated perfused liver (IPRL) system.

(4) Initiating kinetic studies in the IPRL system.

These tasks have been successfully accomplished. Tasks (1) and (2) are applicable to model development and validation of *in vivo* kinetics. Tasks (3) and (4) are related to using the IPRL as an intermediate stage in the development of the *in vitro - in vivo* extrapolation paradigm.

ACCOMPLISHMENTS/NEW FINDINGS

During the first six months the following tasks were completed.

Task (1): To develop a flexible generic BBK model for water soluble diffusion limited (WSDL) chemicals that can be used to simulate kinetics of such chemicals in mammalian systems and to test kinetic hypotheses.

A generic BBK model (**W**ater **S**oluble **D**iffusion **L**imited - **R**at: Version **10** (WSDLR10)) for the kinetics of WSDL chemicals in the rat has been coded in ACSL11 and an executable program has been debugged. The model can be exercised on a 486 computer in the ACSL/Windows environment. The model includes the following biochemical/physiological features:

1. Membrane transport (both simple diffusion and mediated transport)
2. Protein binding
3. Metabolism
4. Biliary/urinary excretion
5. GI absorption and elimination
6. An RBC compartment

Currently WSDLR10 can be employed as a heuristic learning tool to investigate the kinetic behavior of WSDL chemicals in the rat. As experimental data is collected for the test chemicals proposed for this program, the model will be used to analyze the kinetics of these chemicals *in vivo*. Once this model is validated, it can be applied to evaluating the kinetics of WSDL chemicals of interest to the Air Force. The TCA component of this study will have additional Air Force payoffs. The data being collected for TCA (see below) and related kinetic simulations with WSDLR10 are directly applicable to the risk assessment of trichloroethylene (TCE) since TCA is a WSDL metabolite of TCE and may contribute to the cancer promoting effects of TCE in experimental animals.

Task (2): To conduct *in vivo* kinetic studies on trichloroacetic acid, one of the test chemicals included in Phase 1 of the research program.

Two series of kinetic studies with trichloroacetic acid (TCA) have been conducted in collaboration with researchers at WPAFB. The longer term studies (48 h) determined ^{14}C-TCA kinetics in plasma, RBCs, six tissues, urine and feces at 7 time points. Radiochemical analyses of biological samples are complete and HPLC analyses for parent TCA are in progress. The data indicate that the ranking of tissue radiolabel concentrations, in descending order, is plasma > kidney > liver > skin > muscle > fat. The major portion of the radiolabel is excreted in the urine after 24 h. The kinetic data will be analyzed using WSDLR10 to fit the permeability parameters. These permeability values will be compared to those determined in the *in vitro* membrane vesicle system when they become available. A second set of short term (1 h) kinetic data for TCA has been obtained using cannulated rats. These studies are in progress. The purpose of the short term studies is to investigate early distribution of the test chemical between plasma and tissues. These early time data are most useful in evaluating diffusion limited chemicals. Experimentally determined *in vivo* kinetic data form the critical data set to be used for establishment of the *in vitro - in vivo* extrapolation procedure using the three test chemicals in the developmental phase. In this phase, an iterative procedure is employed to optimize the extrapolation procedure. For the second set of three chemicals in the validation phase, the *in vivo* kinetics will be predicted *a priori* on the bases of the experimentally determined *in vitro* data and the BBK model.

Task (3): To develop a generic BBK model for the IPRL to be used to analyze the kinetic data obtained from the IPRL studies.

A generic BBK model for the IPRL system (**I**solated **P**erfused **R**at **L**iver - **R**ecirculating - Version **30** (IPRLR30)) has been coded in ACSL11 and an executable program debugged. The model can be exercised on a 486 computer in the ACSL/Windows environment. The model is designed to take into account the following biochemical/physiological processes in the liver:

1. Membrane transport (both simple diffusion and mediated transport)
2. Protein binding
3. Metabolism
4. Biliary excretion

IPRLR30 corrects for the removal of samples from the system during the course of the experiment. The current version of IPRLR30 describes the behavior of both the parent compound and one metabolite assuming that metabolism can be described by a Michaelis - Menten type equation. Additional metabolic pathways can be incorporated into the model as needed. This model will be used to analyze the kinetic data provided by the IPRL system. The structure of the model for the liver is identical to the liver compartment in the *in vivo* model for WSDL chemicals (WSDLR10). Therefore, model parameters that are determined using data from the IPRL system can be directly incorporated into the *in vivo* biokinetic model without further manipulation.

Task (4): To develop a model system, intermediate between cells *in vitro* and the intact animal *in vivo*, that can be used to explore the role of the liver in controlling the kinetics of xenobiotics.

The isolated perfused rat liver perfusion system has been set up in this laboratory. This system can be used to investigate physiological and biochemical processes in the liver that control the kinetics of xenobiotics. A unique advantage of the IPRL system is that biliary excretion can be investigated. A series of control experiments are in progress using an automated IPRL system developed by the Navy at WPAFB. This system is being modified for the purposes of this research program and 3 hour control perfusions are being conducted to determine baseline biochemical and physiological parameters of the perfused rat liver (GSH concentration, ATP - energy charge, tissue enzyme levels (LDH, AST, ALT), glycogen, lipid oxidation (TBARS), bile production, oxygen consumption and pathology are evaluated). These studies will be used to establish quality control criteria for the kinetic studies to follow.

The IPRL experiments are intermediate studies in the development of the *in vitro - in vivo* extrapolation paradigm. In conjunction with the BBK model developed (Task 3), the kinetics of three test chemicals to be utilized in the first phase of this project will be evaluated to confirm that *in vitro* measurements of kinetic parameters using membrane vesicles can in fact be extrapolated to more complex biological systems. The connections between the *in vitro* membrane vesicle model, the IPRL model and the *in vivo* situation are based on BBK models that are constructed with parallel structures. If the process can be validated in a step-wise manner, then in future studies the intermediate steps can be ignored and *in vivo* kinetics can be predicted solely on the basis of *in vitro* studies.

Personnel Supported

Corike Toxopeus - Ms. Toxopeus is visiting WPAFB for the summer under an EOARD visiting scientist program. Ms. Toxopeus will return to Holland in September to defend her Ph.D. thesis at which time she will be hired as a Postdoctoral Fellow through Wright State University under this research grant. Ms. Toxopeus has been involved in setting up the IPRL system. In addition, she has been conducting baseline measurements of biochemical parameters in perfused rat livers and isolated rat hepatocytes.

John Wyman, Ph.D. - Dr. Wyman is an employee of ManTech Environmental Technology, Inc. at the Toxic Hazards Research Unit, WPAFB. Dr. Wyman has previous experience in the

IPRL system and has assisted in training Ms. Toxopeus in the automated perfusion apparatus and in surgical preparation of the perfused liver.

Hugh Barton, Ph.D. - Dr. Barton is an employee of ManTech Environmental Technology, Inc. at the Toxic Hazards Research Unit, WPAFB. Drs. Frazier and Barton have been collaborating in the collection of *in vivo* kinetic data for TCA.

Publications

Frazier, J.M. (1995) Application of *in vitro* systems to the prediction of *in vivo* biokinetics. *Toxicol. In Vitro*. In press.

Interactions/Transitions

a. Participation/presentations at meetings, conferences, seminars, etc.

Annual Meeting Society of Toxicology, March 5-9, 1995: Presented a poster entitled - Development of a biologically-based kinetic model for aqueous soluble, diffusion limited chemicals.

Invited participant in the European Center for Validation of Alternative Methods Workshop, March 21-23, 1995 entitled - Use of Biokinetics and In Vitro Methods in Risk Evaluation.

Invited lecturer - Western Michigan University, April 11-12, 1995. Lecture entitled - Acute hepatotoxicity of cadmium - A case study in predictive toxicity.

b. Consultative and advisory functions

NA

c. Transitions

NA

NEW DISCOVERIES, INVENTIONS OR PATENT DISCLOSURES

NA

HONORS/AWARDS

NA

APPENDIX 2

A BIOLOGICALLY-BASED KINETIC MODEL FOR THE ISOLATED PERFUSED RAT LIVER.

J M Frazier[1] and C Toxopeus[2]. [1] TriService Toxicology Consortium, Wright-Patterson Air Force Base, OH; [2] Wright State University, Dayton, OH.

The isolated perfused rat liver (IPRL) preparation is a useful tool to investigate the role of the liver in the kinetics of xenobiotics in mammals. This model system is particularly useful because it maintains the liver architecture, as well as the normal route of perfusion. In addition, mechanisms of biliary excretion can be investigated. In order to describe the kinetic behavior of xenobiotics in the system, a generic biologically-based kinetic (BBK) model for the IPRL system has been coded in ACSL11 and an executable program debugged. The model is designed to take into account the following biochemical and physiological processes in the liver: (1) membrane transport, (2) protein binding, (3) metabolism, and (4) biliary excretion. The model corrects for the removal of samples from the system during the course of the experiment. The current version describes the behavior of both the parent compound and one metabolite, assuming that metabolism can be described by a Michaelis - Menten type equation. Additional metabolic pathways can be incorporated into the model as needed. This model can be used to analyze kinetic data for the concentration of the parent chemical and the metabolite in the perfusion medium, in bile and in the liver at the end of the perfusion.